Identification of Human Biomarkers for Cocaine Addiction Diagnosis

Rebecca Romack, April 2008

Abstract

Addiction is a result of long-lasting behavioral changes including tolerance (the need for escalating doses to achieve the same effect) and dependence (physical symptoms manifested during abstinence). It is estimated that 35.3 million Americans have tried cocaine at least once, 6.1 million have used in the past year, and 2.4 million have used it in the past month according to the National Household Survey on Drug Abuse report in 2006, indicating the need for accurate diagnosis of dependence among a large portion of the population. It is important to diagnose cocaine use and dependence by finding definitive markers, termed biomarkers, of such a disease state. For comparison, blood tests for the presence of cocaine are only effective for the 20 minutes to several hours that the drug remains in the body. At present diagnosis of cocaine dependence requires subjective psychological and physical testing and interviews. The goal is to use microarrays to identify a specific set of biomarkers which can reliably determine a diagnosis of cocaine addiction. The impact of this research includes development of novel pharmacotherapies to prevent addiction and relapse and to improve the quality of life and productivity of addicted and recovering individuals and their communities.

This experiment uses global gene expression analysis to identify changes in the RNA of cocaine addicts versus non-addicted individuals. Total RNA is extracted from whole blood samples, purified, hemoglobin transcripts are removed, and RNA is quantified with Nanodrop spectroscopy. The RNA is the hybridized to oligonucleotide microarrays containing 49,000 genes. This project will ultimately produce useful data on diagnostic biomarkers of cocaine addiction. It can help clinicians to diagnose addicts and to determine what drugs a patient has been using long term. The project is a collaboration that will identify correlations between gene expression patterns and subject behavior as reported by UTMB Galveston and UTHSC-Houston,

thus enabling unambiguous diagnosis of addiction. My role in the project is to obtain high quality, ultra pure RNA from whole blood samples, with the goal of discovering biomarkers of cocaine addiction.

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I. Background

Historical Overview

Cocaine is a naturally-occurring alkaloid from the leaves of *Erythroxyyon coca*, a plant whose leaves, shown in Figure 1, have been chewed by South Americans for approximately 5000 years (Feldman et al, 1997). Cocaine was chemically extracted from the leaf in 1859, after which it was used in many substances. There are several forms of cocaine, including the raw leaf, a crystalline powder, and the free base form, crack cocaine. Purified cocaine is stable as a hydrochloride salt that forms a crystalline powder, shown in Figure 2(a), which is usually snorted or injected. Pure cocaine is also stable as the free base form (including crack cocaine, shown in Figure 2(b)), which can be smoked.

Figure 1: Leaves of *Erythroxyyon coca*

Figure 2. (a) Cocaine powder (b) Crack cocaine

Historically unregulated cocaine-containing formulations included: Coca-Cola, originally prepared with a recipe that called for cocaine extract, cocaine toothache drops marketed at very young, teething children for cocaine's anesthetic properties, and Vin Mariani, endorsed by Pope Leo XIII, who carried a hip flask of this wine, (Figure 3). After increasing popularity of the use of cocaine, its addictive properties were realized. Consequently, in 1914, the Harrison Narcotics Tax Act made the sale of cocaine illegal in the United States. However, there are some legal uses of cocaine today, such as in topical anesthetics (Aldous et al, 1998). Cocaine, along with benzocaine, novacaine, and lidocaine, cause local anesthesia by blocking voltage gated sodium channels in the neuron plasma membrane (Ahern et al, 2008). Cocaine acts as a topical anesthetic at high doses (4% cream applied locally, Aldous et al 1998), and was traditionally used in eye and nasal surgery for this reason.

(a) (b) (c)

Figure 3: (a) Coca-Cola advertisement, (b) Cocaine toothache drops, (c) Vin Mariani endorsement.

Medical and Behavioral Effects

Cocaine is a psychomotor stimulant, which means with short term use it increases alertness as well as motor activity (Feldman et al, 1997). Cocaine also causes local anesthesia due to the blockage of sodium channels. With long term use, cocaine induces behavioral changes including compulsive, stereotyped motor activity such as head bobbing and tongue movement, psychotic behavior similar to schizophrenia, and hallucinations. One common hallucination is of insects crawling under the skin; users will scratch and cut themselves to get the “crack bugs” out (Cesar). Other long term effects of cocaine include a high risk of cerebro-ventricular accident or stroke and convulsions (Koppel et al, 1996).

Cocaine addiction is a disease characterized by dependence, which, defined for alcohol, is diagnosed by the presence of three or more subjective symptoms (DSM-IV). They include: the need for more drug for same effect (tolerance), craving or withdrawal syndrome or use of the drug to prevent withdrawal symptoms, desire or effort to reduce usage, using more than intended, important lifestyle activities reduced by drug use, significant time spent using or

recovering from using, and drug use despite physical and/or psychological problems directly caused by the drug. These criteria can be broadly applied to all addictive substances.

Cocaine dependence causes lethargy, depression, and an increased probability of fatal overdose (Feldman et al, 1997). Cocaine acts very quickly to achieve a high, and the low similarly occurs rapidly. Dysphoria leads to craving for more cocaine soon after use by an addict, which in turn leads to multi-dose binges lasting hours. Cocaine is rewarding in the short term because it blocks monoamine transporters in brain regions important in reward and reinforcement. This blockage prolongs neurotransmitters' action at their receptors. The primary neurotransmitter involved in these brain regions is dopamine. This brain imbalance, in the long term, has drastic consequences for the health of the user.

Eighty-three million dollars was spent on hospitalization of cocaine users in 1995, with the most common symptom being chest pain associated with myocardial infarction (Drug Abuse Warning Network, 2001). In 2000, approximately 175,000 cocaine-related visits were made to an emergency department in the United States (DAWN, 2001). In addition, cocaine dependence can be passed on to a fetus in the womb. Each addicted baby born of an addicted mother costs medical and social systems $750,000 to $1.5 million (Kalotra, 2002). Obviously, accurate diagnosis of cocaine addiction is important.

Molecular Effects: Gene Transcription and Expression

How does cocaine cause such diverse clinical aspects, as outlined above? The primary neurochemical effect of cocaine dosage is increased synaptic concentrations of the reward neurotransmitters dopamine and serotonin, and the stimulatory neurotransmitters norepinephrine and epinephrine (Feldman et al, 1997). Cocaine blocks all these monoamine transporters, preventing reuptake of neurotransmitters for recycling. The psychomotor effects of cocaine are specifically associated with dopamine systems. The dopamine transporter and the structure of cocaine and of dopamine are illustrated in Figure 4 to indicate their interaction. Addiction alters neuronal transmission and synaptic structure and organization through neurotransmitters, sometimes permanently (Figure 5).

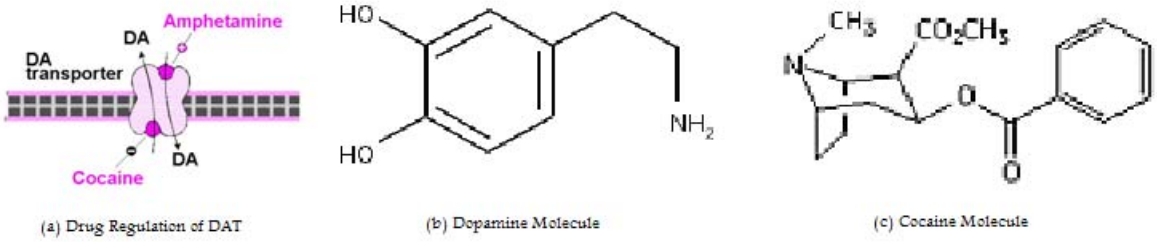


Figure 4. (a) Dopamine transporter (b) Dopamine molecule (c) Cocaine molecule
Note the structural similarities of dopamine and cocaine, especially the benzene ring and the amine groups.

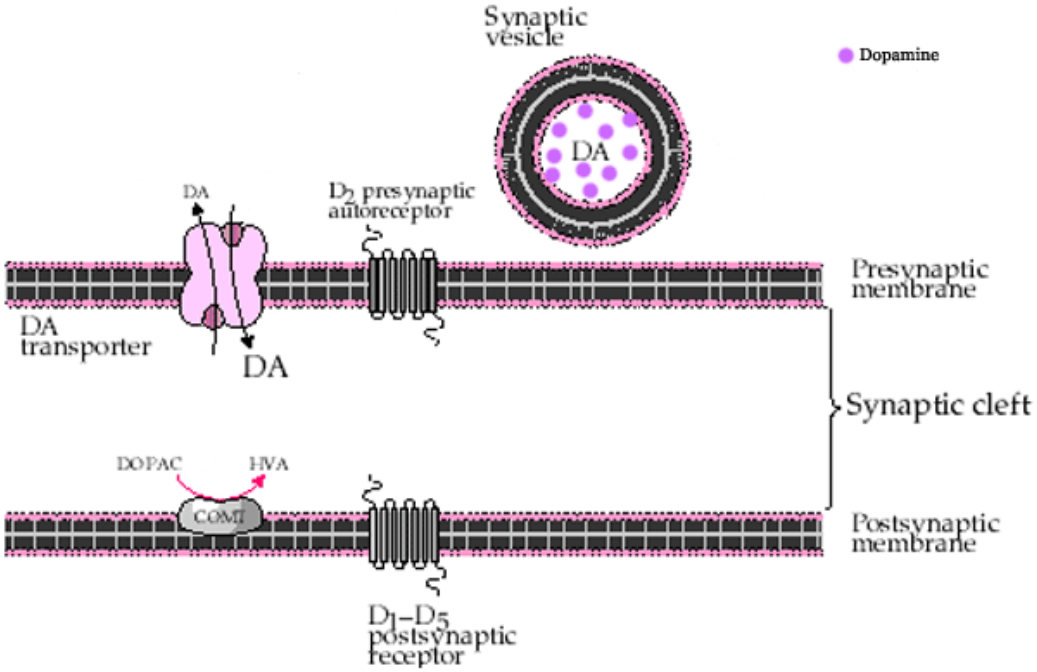


Figure 5. Organization and regulation of dopamine transporter and receptors

Furthermore, neurotransmitters and neuromodulators activate receptors, which can then activate second messengers such as calcium and cAMP (cyclic adenosine 3,5-monophosphate) (Dube and Amireault, 2007). Second messengers can affect gene expression by activating transcription factors such as CREB (cAMP response element binding protein) to enhance or suppress transcription of DNA. In this way, cocaine and other drugs of abuse can alter gene expression.

Cocaine responsive genes have previously been identified in mouse brain studies (Freeman et al, 2007). The categories of genes were involved in neurological signaling pathways. These genes are transcribed from DNA to messenger RNA transcripts, which in turn are translated to proteins with diverse functions which ultimately play a significant role in cellular function (Hartwell et al, 2004). The expression of genes is primarily regulated at the point of

initiation of transcription. Proteins called transcription factors bind to the DNA for regulation. Messenger RNA for proteins is encoded by Class II genes, which contain regulatory regions called promoters and enhancers. Promoters are located at the 5' end (the phosphate end) of the gene of interest, whereas enhancers may be located in a number of regions with respect to their gene. Regulation of transcription depends on interaction of multiple enhancers with competing transcription factors. This integration of signals determines the precise level of transcription of a specific gene in a specific cell at a specific time (Hartwell et al, 2004).

Genetic Factors

In addition to altering genetic expression, cocaine addiction has a genetically inherited component. Genetic factors are estimated to account for 40-60% of addiction risk (Nestler, 2000). One important goal is identification of human genetic and biological traits that diagnose addiction. Quantitative trait locus analysis in animal models can be used to map behaviors to specific genes. Some researchers are searching for genes of susceptibility to addiction as well. Once discovered, these genes are investigated in animal and other models. Of course, the complex trait of addiction involves many genes, each contributing some small effect to what can be an overwhelming behavioral problem. However, these behaviors are not always easy to quantify.

Biomarkers

Short-term biomarkers can easily be quantified by urinalysis and other tests. Short-term biomarkers of cocaine use, such as the pyrolyzate metheylecgonidine and its metabolite ecgonidine have half-lives of 18-21 minutes and 94-127 minutes, respectively (Scheidweiler, 2003). These molecules (Figure 6) are specifically associated with smoking cocaine only, due to the heat-activated conversion of cocaine to methylecgonidine. Methylecgonidine is an in vitro muscarinic agonist, which means it stimulates the production of nitric oxide in cardiac tissues, causing the smooth muscle of blood vessels to relax, and permitting vasodilation to increase blood flow. This biomarker can be found in urine, saliva, and perspiration, but its short life is it main disadvantage.

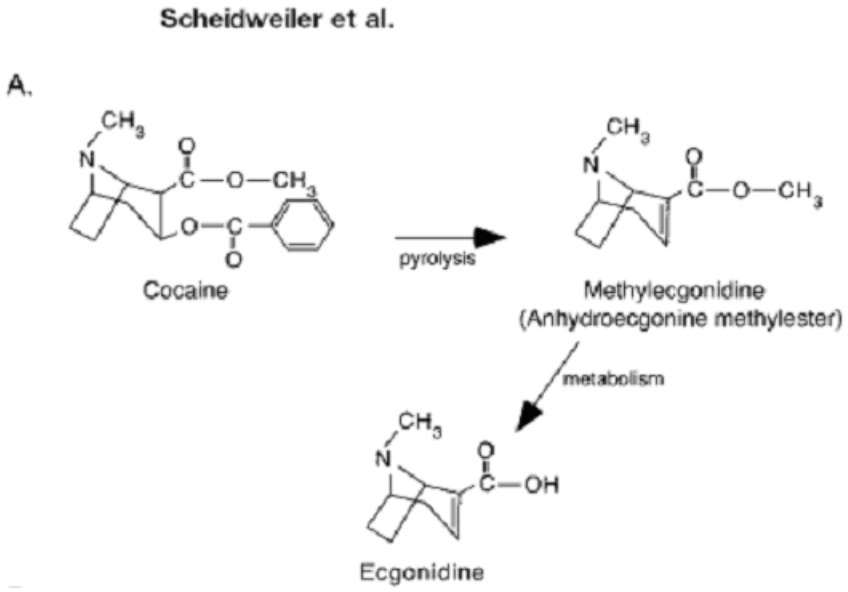


Figure 6. Short-term biomarkers of cocaine smoking (Scheidweiler, 2003)

Cocaine dependence is a complicated condition, and as such, a single biomarker is unlikely to serve as a reliable diagnostic indicator. It is important to use RNA to identify biomarkers of addiction (a biomarker is any molecule, whether protein, RNA, or other

metabolite, that acts as a definitive signal of a particular disease state), because RNA levels are indicative of upregulation and downregulation of genetic expression. A specific pattern of changes in gene expression may act as a diagnostic biomarker. This experiment aims to discover such a pattern using RNA from whole blood samples from cocaine dependent individuals. Human erythrocytes lack nuclei, so the vast majority of RNA is from the leukocytes.

Animal studies have shown that even after long-term abstinence (30 or 100 days), cocaine reinforcement persists due to changes in RNA expression levels (Freeman, 2007). These results demonstrate altered gene expression after cocaine administration and abstinence. Changes in gene expression persist long after cessation of drug consumption, and may be associated with repeated relapses among addicted individuals. Regulation of gene expression, including chromatin modification, changes an addict for a long time, sometimes a lifetime.

II. Methods

Experimental Design

This experiment will identify transcriptional changes to diagnose the disease state of cocaine addiction. Blood samples were supplied by the University of Texas Medical Branch in Galveston and the University of Texas Health Science Center at Houston. Multiple drug use is a common problem among cocaine dependent individuals. The 116 patients in the clinical study can be categorized by:

- Cocaine dependence (only)
- Cocaine dependence with alcohol dependence
- Cocaine dependence with Cannabis use
- Cocaine dependence with opiate dependence

RNA Extraction from Whole Blood

This experiment uses global gene expression analysis to identify changes in RNA levels of cocaine addicts versus non-addicted individuals. Total RNA is extracted from whole blood samples, purified, hemoglobin transcripts are removed, and RNA is quantified with Nanodrop spectroscopy. The goal is to obtain high quality, ultra pure RNA from whole blood samples, to identify patterns of gene expression that may be used as biomarkers.

Four milliliter blood samples are obtained from patients enrolled in ongoing clinical trials. Total RNA is extracted and purified with a PAXgene Blood RNA Kit shown in Figure 7 (Qiagen, USA) through centrifugation, resuspension, and incubation with proteinase, then DNase to remove all cellular fractions that lack RNA. Nanodrop spectroscopy quantifies the RNA, which is critical because 1 μg is required for microarray hybridization. The 260/280 and the

260/230 ratios reveal the RNA content of the elution.

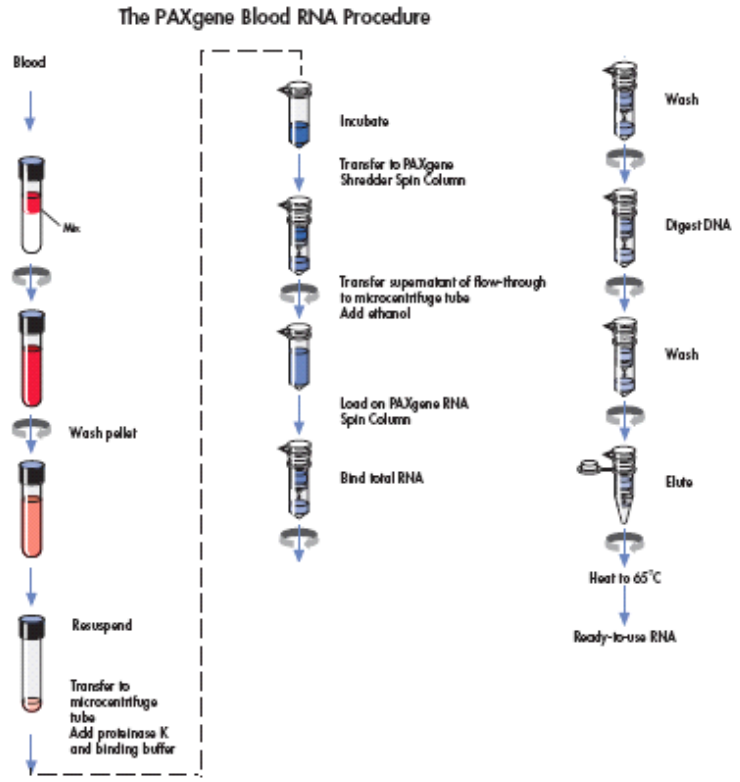


Figure 7. PAXgene RNA extraction from blood (Qiagen)

Globinclear Transcript Removal

Approximately 70% of the RNA in human blood is hemoglobin mRNA, which dilutes the other transcripts and produces a saturated signal if used for microarray analysis. Thus, after ethanol precipitation to concentrate the solution, the globin transcripts were removed using a commercially available kit shown in Figure 8 (GLOBINclear kit from Ambion, USA). By removing globin transcripts, the signal lost is compensated by the magnitude of signal gained for every other transcript. The hemoglobin RNA is biotinylated, and the biotin binds magnetic beads. The beads contain iron, and are collected by placing the microfuge tubes on a magnetic stand. After GLOBINclear the RNA is analyzed by Nanodrop spectroscopy, then its quality was

examined using an Agilent BioAnalyzer to ensure pure, intact RNA for microarray hybridization. Next, the BioAnalyzer requires exactly one micro liter of sample in a Nano chip with various dyes to determine the RNA integrity number (RIN), which quantifies any degradation the RNA may have experienced. An electropherogram conveys visual information about the RNA quality.

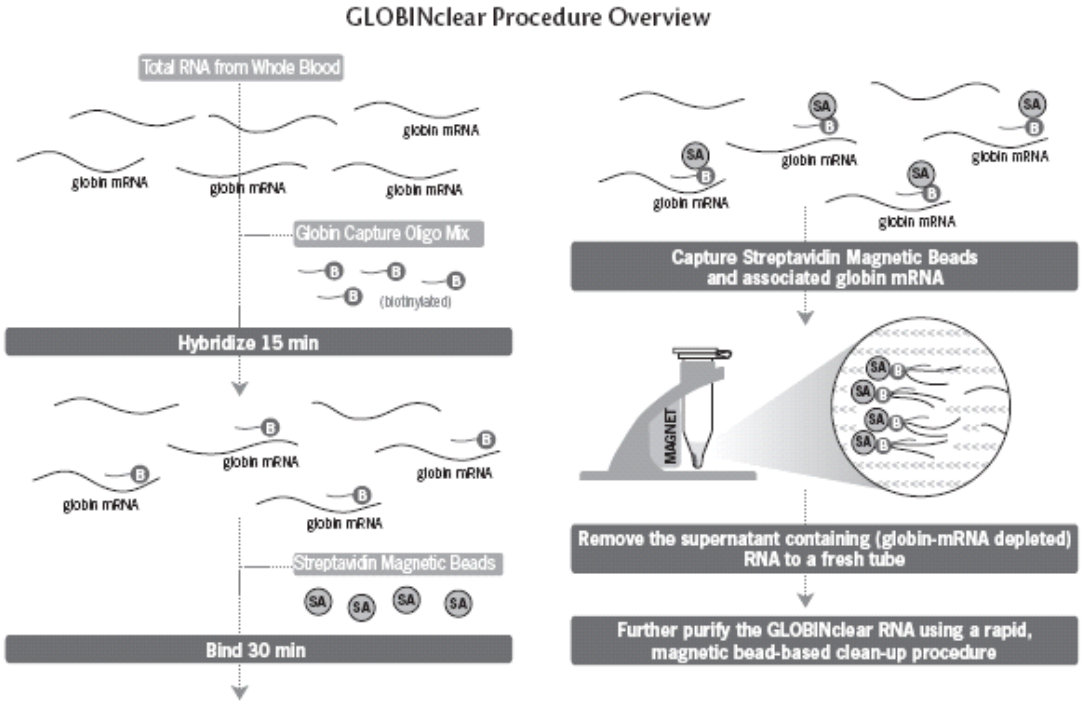


Figure 8. GLOBINclear Protocol (Ambion, Inc., USA)

Microarray Hybridization

The RNA is reverse transcribed to complementary DNA, and hybridized to an oligonucleotide microarray which detects approximately 47,000 genes of known sequence. The controls include non-addicted individuals' RNA, as well as the Universal Human Reference RNA. After scanning the array, each spot is red or green due to fluorescent dye, and the

brightness is quantified.

A DNA microarray is an ordered array of nucleic acids that enables parallel analysis of complex biochemical samples (Schena et al, 1995). This tool for understanding genes and mutations involves a collection of microscopic spots of DNA attached to a glass, plastic, or silicon forming an array for the purpose of monitoring expression levels for thousands of genes simultaneously (called expression profiling). In a spotted microarray, the probes are oligo DNA or 70 base sequences. The array is hybridized with DNA from two samples to be compared, such as the experimental sample and a Universal Human Reference RNA, labeled with two different colors of fluorescent dye. The samples are mixed and hybridized to the microarray, which is then scanned, allowing visualization of up-regulated and down-regulated genes at the same time.

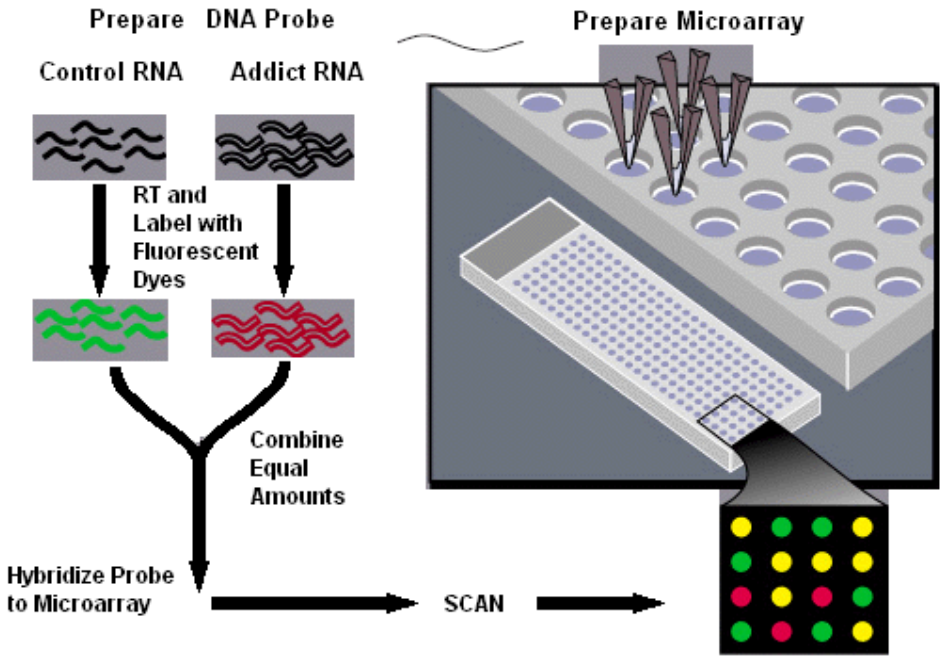


Figure 9. Theory of Microarray technology. Image from “Chemistry Online: An Approach Based on Chemical Logic.”

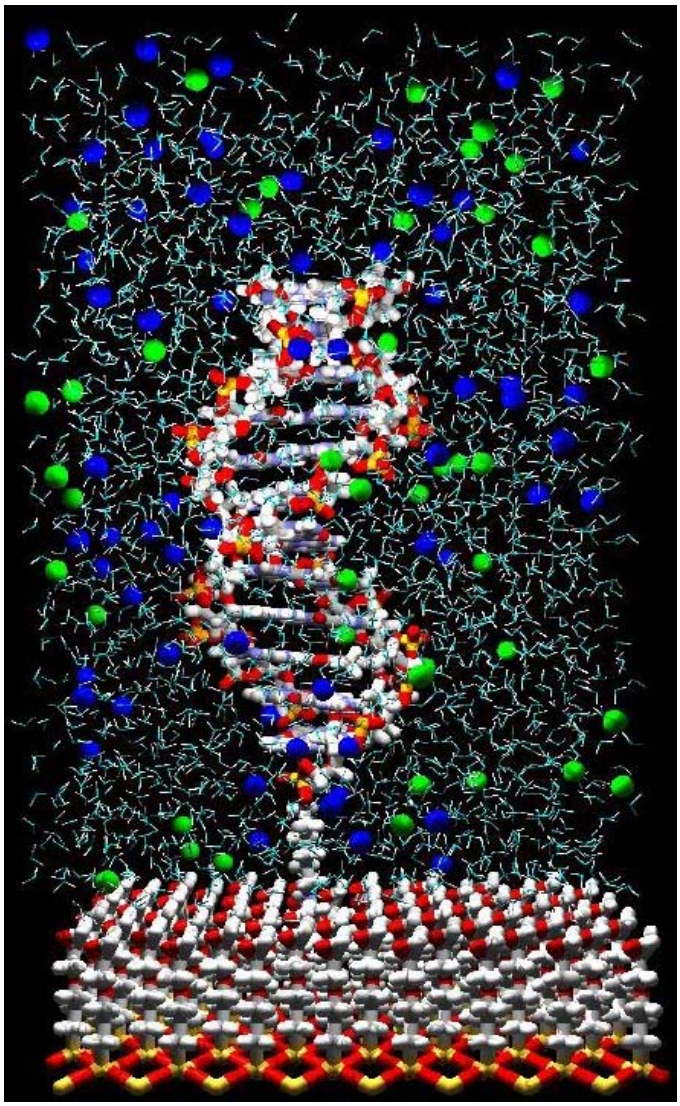


Figure 10. DNA hybridization to a microarray.

On the molecular level, DNA binds to the oligo DNA on the microarray plate at complementary nucleotide sequences. Image from National Partnership for Advanced Computational Infrastructure, 2000.

III. Results

Clinical Information

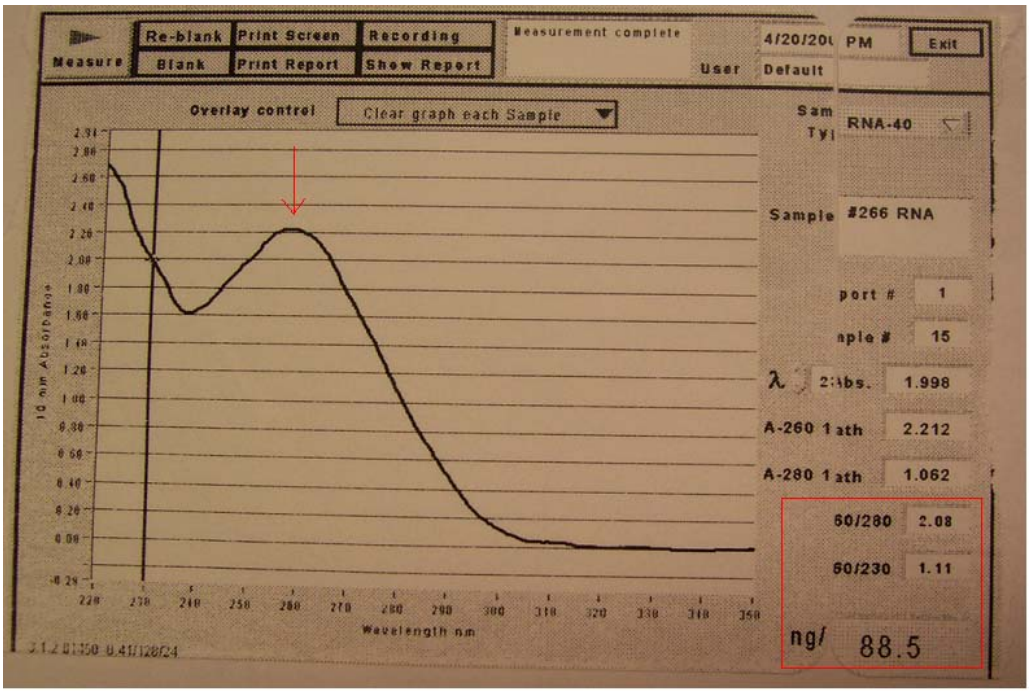
One hundred and sixteen blood samples had RNA extracted and purified for downstream microarray hybridization, which is currently taking place. Blood samples were collected in duplicate to ensure a sufficient quantity of RNA. The samples varied in quality and quality, and identical samples were often pooled for microarray hybridization. The hemoglobin transcripts were removed, and the purity of RNA was tested on the Bioanalyzer from Agilent. It has yet to be determined what the cutoff should be for an acceptable RIN, but higher is better. As shown in the post-GLOBINclear data in Figure 11, multi-drug use is common among cocaine dependent individuals. Clinical diagnosis was based on a battery of psychological tests. These patients were included in a list of de-classified diagnoses sent by UTHSC-Houston, and are representative of the hundreds of extracted and purified samples of RNA in this experiment.

Sample ID	RNA (ng/ul)	Total RNA (ug)	RIN	Diagnosis
10461 #1	81.9	3.24	8.00	Cocaine Dependence
10461 #2	58.32	3.49	7.90	Cocaine Dependence
10479 #1	51.17	2.13	7.70	Cocaine/Alcohol Dependence
10479 #2	90.87	4.54	8.10	Cocaine/Alcohol Dependence
10481 #1	19.8	2.18592	*n/a*	Cocaine Dependence
10481 #2	8.1	1.0854	*1.1*	Cocaine Dependence
10484 #1	28.15	3.25414	8.3	Cocaine Dependence
10484 #2	29.39	3.503288	8.4	Cocaine Dependence
10485 #1	22.95	3.1212	8.1	Cocaine Dependence/Antisocial Person
10485 #2	7.92	0.899712	8.4	Cocaine Dependence/Antisocial Person
10486 #1	8.45	1.1154	7.9	Cocaine Dependence, moderate; Alcohol
10486 #2	21.44	2.718592	7.8	Cocaine Dependence, moderate; Alcohol
10488 #1	73.94	4.81	7.2	Cocaine/Alcohol Dependence
10488 #2	54.64	3.38	5.9	Cocaine/Alcohol Dependence
10489 #1	63.21	4.02	7	Cocaine Dependence, severe; Cannabis
10489 #2	22.52	1.37	7.3	Cocaine Dependence, severe; Cannabis
10497 #1	70.5	8.3754	5.7	Cocaine Dependence, severe; Cannabis
10497 #2	32.1	4.09596	n/a	Cocaine Dependence, severe; Cannabis

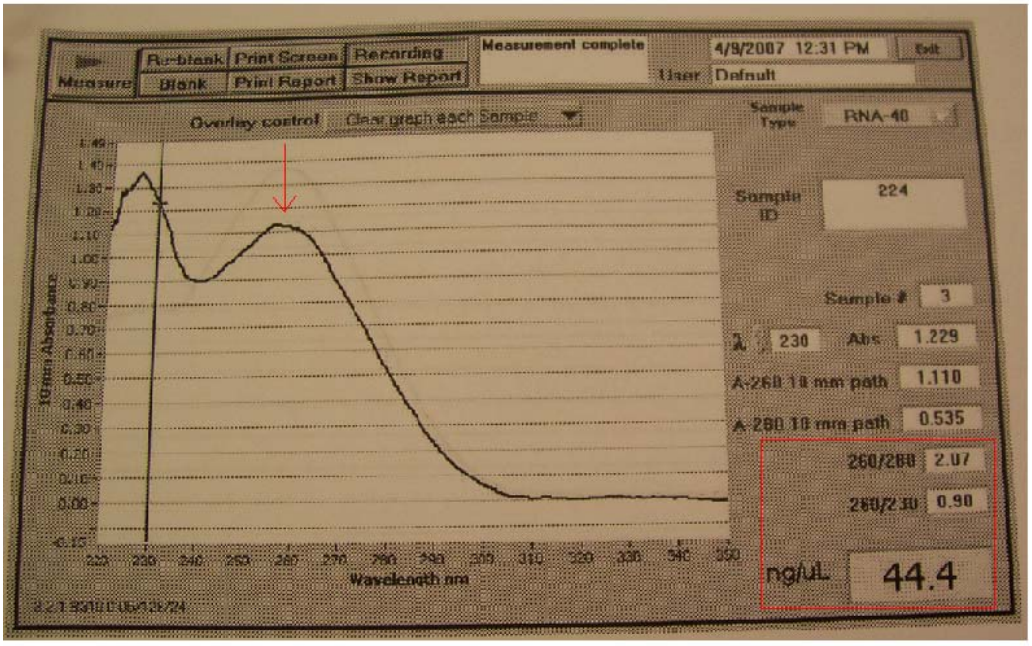
Figure 11: Sample of de-classified clinical data from the originally double blind study.

Upon initial extraction from whole blood, the quantity of RNA was typically several micrograms (Figure 12 shows two representative samples which are followed throughout the entire methodology). RNA was concentrated by ethanol precipitation, then the RNA pellet was resuspended in a smaller volume of nuclease-free water for stable storage. A dilution of this volume was measured with Nanodrop spectroscopy, as shown in Figure 13. After GLOBINclear, whether due to degradation over time, microscopic impurities, or due to removal of globin transcripts directly, RNA quantity may decrease below the 1 µg limit required for microarray hybridization (Figure 14).

For each sample the 260:280 and 260:230 absorbance ratios were recorded. If the 260:280 ratio is below 1.8, the sample is likely contaminated with protein, phenol, or other organic reagents used in isolation of the nucleic acid (Thermo Scientific, 2008). The 260:230 ratio is a secondary measure of purity of nucleic acids, and a ratio lower than 2.0 may indicate the presence of EDTA or carbohydrates in the sample. The peak concentration on each absorbance diagram, occurs at 260 nm, indicating that nucleic acid is indeed the predominant component of the samples.

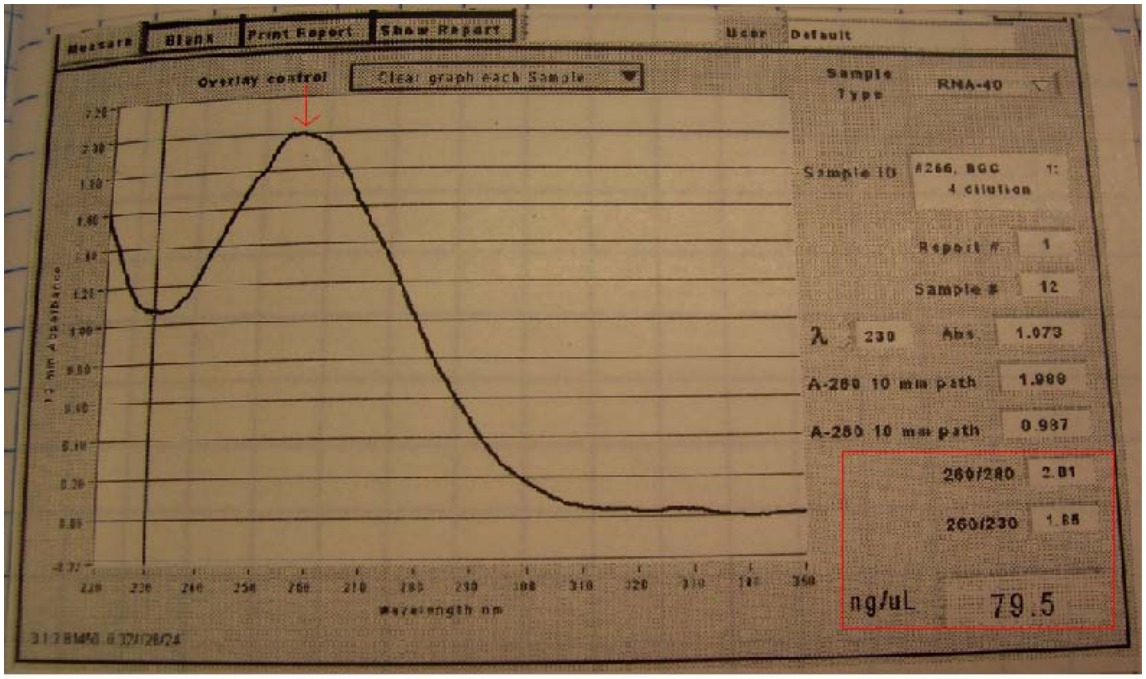


(a)

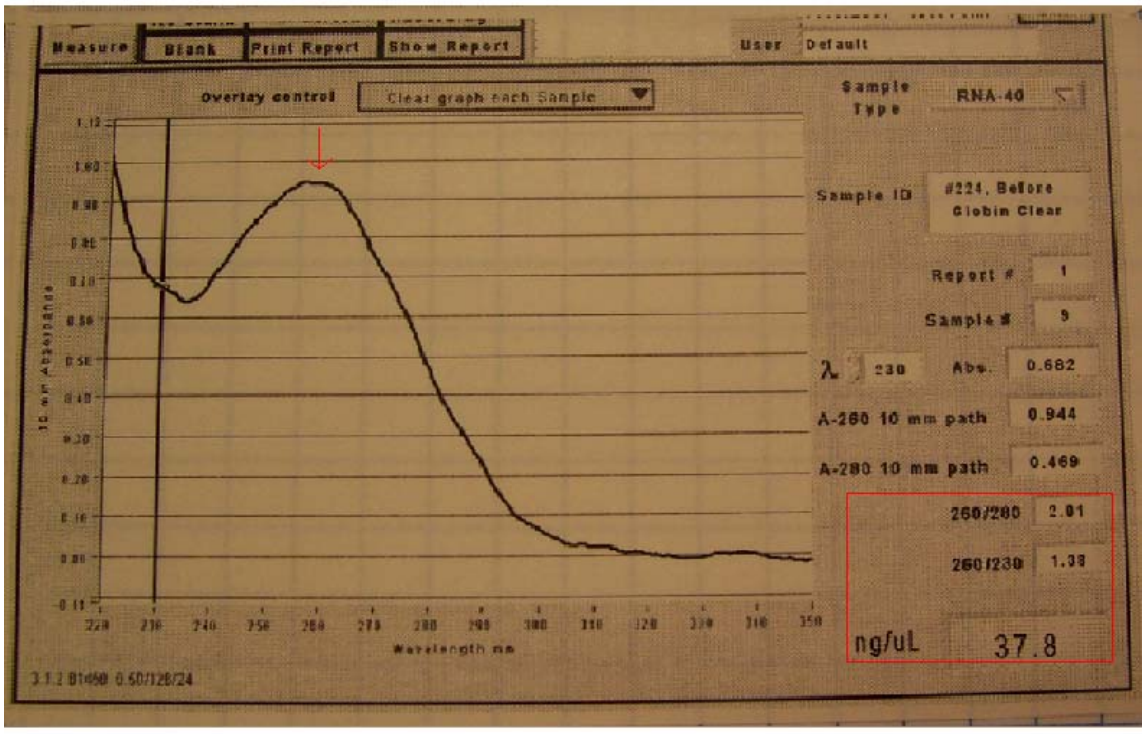


(b)

Figure 12. (a) Sample 266 after extraction (b) Sample 224 after extraction. The initial 260/280 and 260/230 ratios are comparable and of good quality. The 260/280 and 260/230 ratios are indicated in the red boxes, and the peak concentration, indicated by a vertical red arrow, is always at 260 nm. Thus nucleic acid is the major component of the samples.

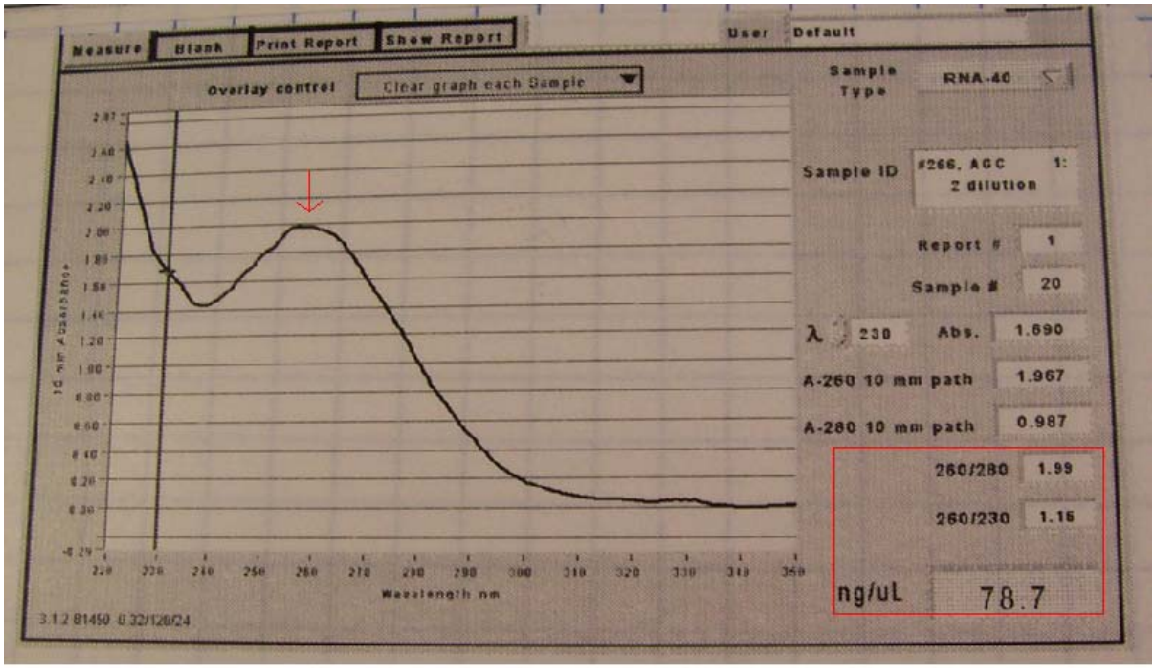


(a)

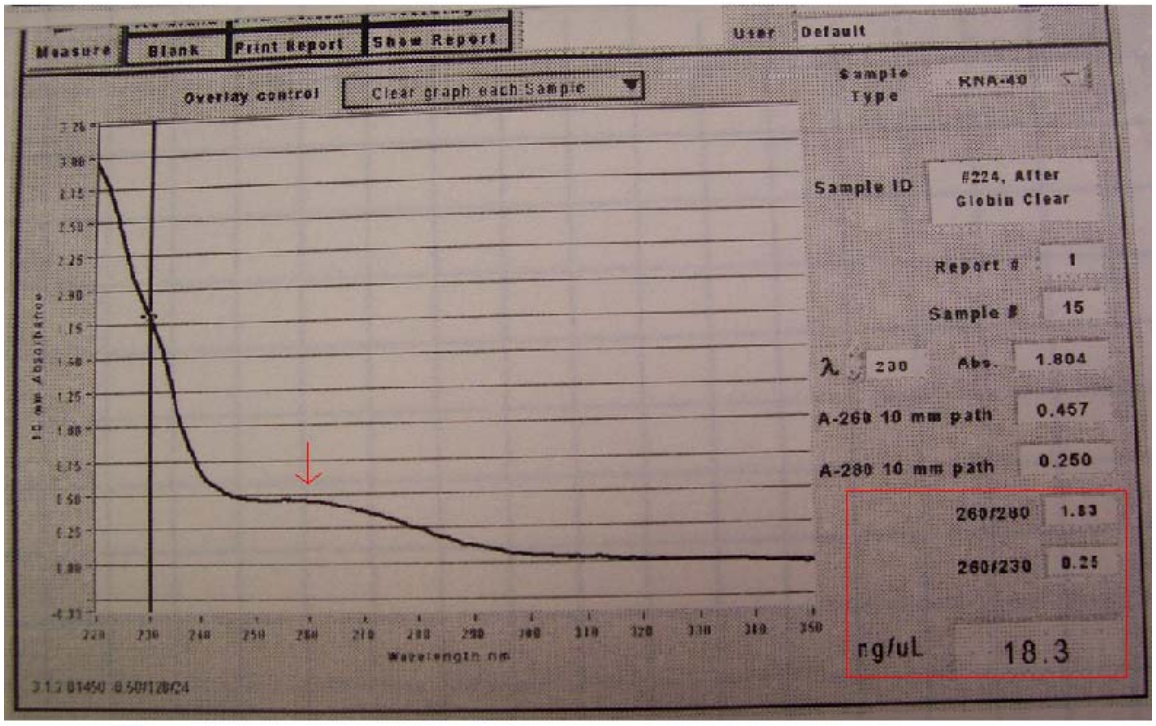


(b)

Figure 13. (a) Sample 266 before GLOBINclear (b) Sample 224 before GLOBINclear. Some RNA is lost when prepared for ethanol precipitation, but the vast majority is still available.

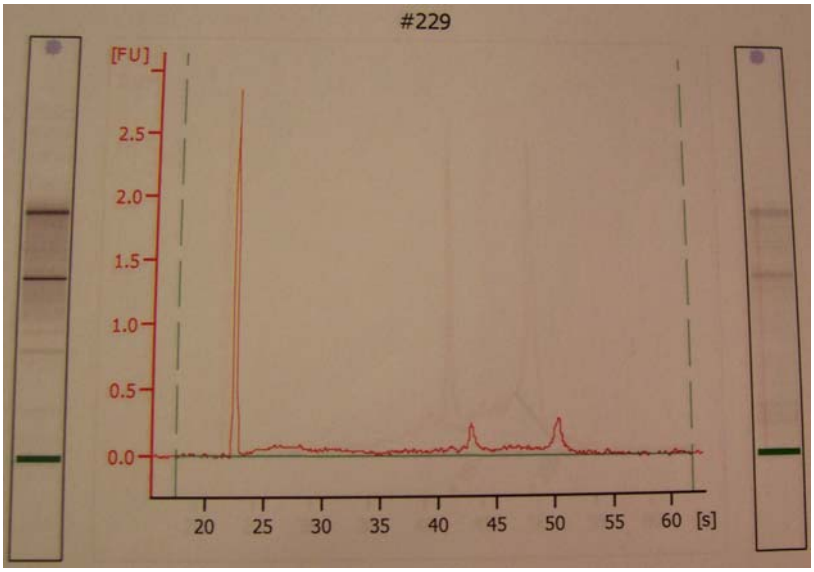


(a)

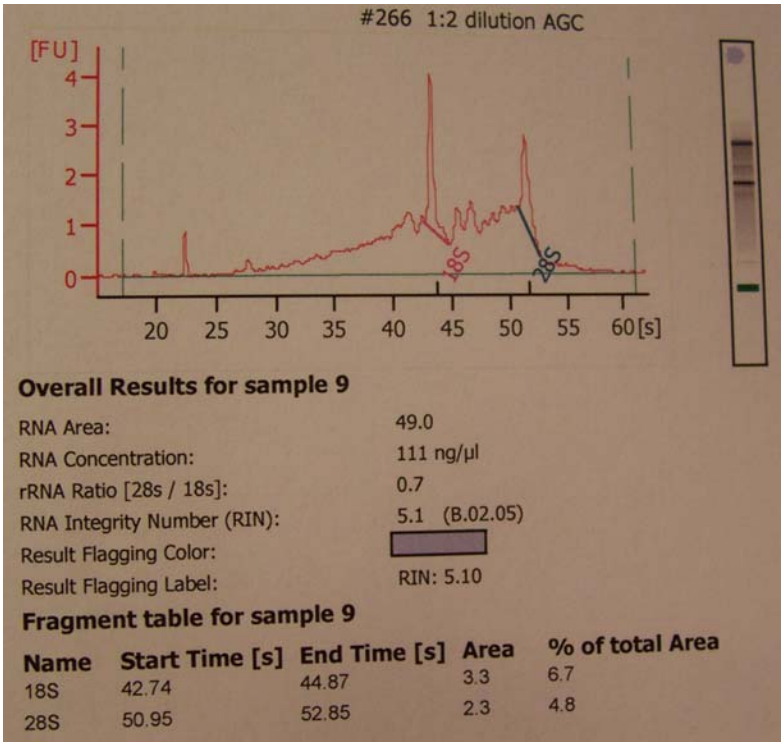


(b)

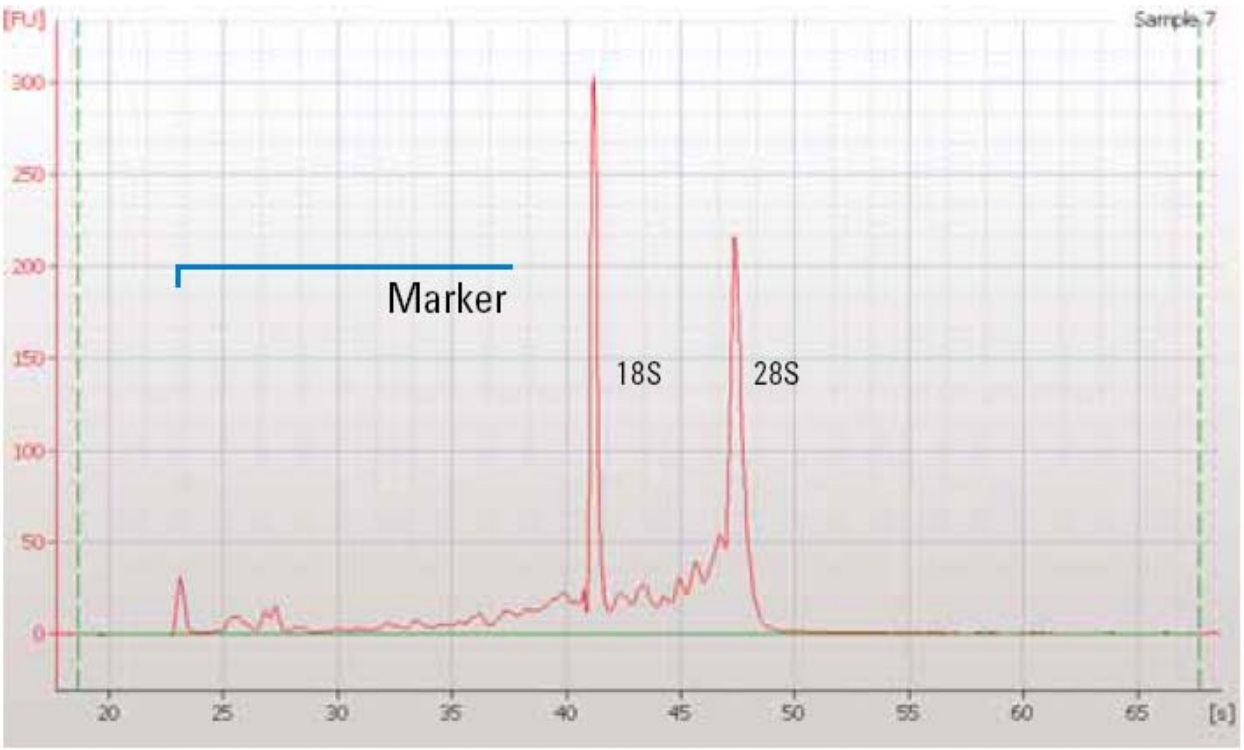
Figure 14. (a) Sample 266 after GLOBINclear (b) Sample 224 after GLOBINclear, now unusable due to degradation and loss of RNA.


#229
[FU]
2.5
2.0
1.5
1.0
0.5
0.0
20 25 30 35 40 45 50 55 60 [s]

(a)


#266 1:2 dilution AGC
[FU]
4
3
2
1
0
18S
28S
20 25 30 35 40 45 50 55 60 [s]
Overall Results for sample 9
RNA Area: 49.0
RNA Concentration: 111 ng/µl
rRNA Ratio [28s / 18s]: 0.7
RNA Integrity Number (RIN): 5.1 (B.02.05)
Result Flagging Color:
Result Flagging Label: RIN: 5.10
Fragment table for sample 9
Name Start Time [s] End Time [s] Area % of total Area
18S 42.74 44.87 3.3 6.7
28S 50.95 52.85 2.3 4.8

(b)



(c)

Figure 15. (a) Sample 229 electropherogram (b) Sample 266 electropherogram

(c) Ideal sample electropherogram (Agilent, USA)

Each peak in the electropherogram represents a subunit. In the ideal sample, the marker peak is of significant height, but small compared to the 18S and 28S subunits (Figure 15 c). This is true of sample #266 as well, Figure 15 b, which is of good quality. However, for sample 229, the 18S and 28S subunits form small peaks relative to the marker, indicating degradation of RNA (Figure 15 a). The yield of pure RNA after purification by GLOBINclear ranged from 3.553 to 7.946 micrograms per blood sample (where duplicate samples were taken from each patient), and the RNA integrity number, on a scale from 1 to 10, with 10 being the highest quality, ranged from 1.10 to 8.80, or from degraded to high quality RNA.

IV. Discussion and Applications

The need for diagnostic biomarkers of addiction is evident. Early diagnosis can permit initiation of treatment before the disease progression becomes severe. A quantitative test for cocaine dependence can help those who might otherwise slip through the cracks of our healthcare system. This project will ultimately produce useful data on such biomarkers of cocaine addiction. It can help clinicians to diagnose addicts and to determine what drugs a patient has been using long term.

The project is a collaboration that will discover correlations between gene expression patterns and subject behavior as reported by UTMB Galveston and UTHSC-Houston, thus enabling unambiguous diagnosis of addiction. As part of the Mayfield lab, my work in RNA extraction and purification has provided hundreds of samples of transcripts from cocaine dependent individuals, including multi-drug users. These samples are used for ongoing microarray hybridization and will eventually contribute to publication. Preliminary findings of differentially expressed genes in cocaine-dependent versus cocaine and alcohol dependent individuals (thus alcohol-related gene expression) included immune response signals, cell adhesion molecules, and the GABA alpha 6 receptor. GABA is the major inhibitory neurotransmitter in the brain (Mitchell et al, 2008). At this point, more research is necessary to perform additional microarray hybridization and to statistically analyze existing information. However, it is hypothesized that significant changes in biomarkers in blood will be found. Although brain tissue studies reveal relatively small, 20-50% changes in gene expression due to disease states (such as alcoholism, Liu et al, 2004), blood sample studies often indicate larger, 150% changes in gene expression levels (Sekiguchi et al, 2008).

The impact of this research includes accurate biochemical diagnosis of addiction or

susceptibility. Early diagnosis is critical to initiating treatment of at-risk individuals. Armed with a set of specific biomarkers of cocaine dependence, clinicians could predict disease and treatment results based on previous patients' successes.

Identification of previously unsuspected biomarkers could reveal novel targets for pharmacotherapy development. In this manner normal levels of transcripts could be maintained in spite of the tolerance and craving experienced by addicted individuals struggling to quit using cocaine. Such drug therapy can make addiction a treatable, manageable disease, and control relapse to improve the quality of life and productivity of addicted and recovering individuals and their communities.

V. References

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